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Suppression of MicroRNA *let-7a* Expression by Agmatine Regulates Neural Stem Cell Differentiation

Juhyun Song[1*], Yumi Oh[1,2*], Jong Youl Kim[1], Kyoung Joo Cho[1,2], and Jong Eun Lee[1,2]

[1]Department of Anatomy, Yonsei University College of Medicine, Seoul;
[2]Brain Korea 21 Plus Project for Medical Sciences and Brain Research Institute, Yonsei University College of Medicine, Seoul, Korea.

Purpose: Neural stem cells (NSCs) effectively reverse some severe central nervous system (CNS) disorders, due to their ability to differentiate into neurons. Agmatine, a biogenic amine, has cellular protective effects and contributes to cellular proliferation and differentiation in the CNS. Recent studies have elucidated the function of microRNA *let-7a* (*let-7a)* as a regulator of cell differentiation with roles in regulating genes associated with CNS neurogenesis.
Materials and Methods: This study aimed to investigate whether agmatine modulates the expression of crucial regulators of NSC differentiation including DCX, TLX, c-Myc, and ERK by controlling *let-7a* expression.
Results: Our data suggest that high levels of *let-7a* promoted the expression of TLX and c-Myc, as well as repressed DCX and ERK expression. In addition, agmatine attenuated expression of TLX and increased expression of ERK by negatively regulating *let-7a*.
Conclusion: Our study therefore enhances the present understanding of the therapeutic potential of NSCs in CNS disorders.

Key Words: Neural stem cell (NSCs), microRNA *let-7a* (*let-7a)*, agmatine, DCX, TLX, ERK

INTRODUCTION

Neural stem cells (NSCs) maintain multipotency and are capable of self-renewal;[1] and it has been demonstrated that dividing NSCs in the subventricular and subgranular zone[2] can become neurons.[3] NSCs have been studied extensively as promising novel treatments for central nervous system (CNS) disorders.[4] Several studies have demonstrated that proliferation of endogenous NSCs is enhanced after brain injury, as is migration of neural progenitor cells to the brain lesion.[5] In addition, NSCs migrate toward injured brain regions, differentiate into neurons, and then facilitate injury healing.[6] Thus, NSC self-renewal and proliferation are important characteristics for the enhanced therapeutic efficiency of NSCs in the brain.[7]

Agmatine is an endogenous amine produced by decarboxylation of L-arginine by arginine decarboxylase present in glia and neurons,[8] and it has been reported to affect various cellular processes, such as antioxidative pathways,[9] and also has a role in CNS injuries including neurotrauma and animal models of ischemia.[10,11] Moreover, agmatine initiates early neurogenesis and cell proliferation.[12]

MicroRNAs can regulate target mRNAs via translational inhibition or mRNA degradation.[13] Recent studies have focused on the mechanisms of microRNA action on NSCs, in order to discover potential treatments for CNS diseases.[14] MicroRNA *let-7* (*let-7*) is a family of microRNAs present in multiple genomic locations,[15] and consists of 9 members.[16] *let-7* targets cyclin D1, and its overexpression promotes cell cycle exit and differentiation.[17] Furthermore, *let-7* suppresses differentiation of human embryonic stem cell derived neural progenitor cells,[18] and influences self-renewal of NSCs.[19] Recent studies have suggested a function of *let-7* in cell fate mechanisms in neurons.[20,21] In addition, overexpression of *let-7a*, one of the members of *let-7* family, influences NSC proliferation and differentiation.[22,23] However, the mechanisms of action of the *let-7* family on NSC proliferation or neuronal differentiation are poorly under-

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Corresponding author: Dr. Jong Eun Lee, Department of Anatomy, Brain Korea 21 Plus Project for Medical Sciences, Yonsei University College of Medicine, 50-1 Yonsei-ro, Seodaemun-gu, Seoul 03722, Korea.
Tel: 82-2-2228-1646, Fax: 82-2-365-0700, E-mail: jelee@yuhs.ac

*Juhyun Song and Yumi Oh contributed equally to this work.
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stood and are debatable. Agmatine has been shown to block neuronal nitric oxide synthase,[10,24] inducible nitric oxide synthase[25] and N-methyl-D-aspartate receptor channels.[26] Furthermore, the nitric oxide synthase inhibitor asymmetric dimethylarginine increased the levels of microRNAs such as microRNA-21.[27] Taken together, therefore, it is quite possible that there exists a relationship between agmatine and *let-7a* in the regulation of NSC differentiation. Accordingly, the aim of the present study was to investigate the effects of agmatine on NSCs by controlling *let-7a* levels.

MATERIALS AND METHODS

NSC primary culture

Cortical NSCs were obtained from pregnant imprinting control region mice (E13.5). The cortices were dissected and washed 1–2 times with Hank's Balanced Salt Solution (HBSS; HyClone Laboratories, South Logan, UT, USA). To each piece of washed tissue, 5 mL of HBSS were added, and the tissue was dissociated by pipetting up and down. Tissues were triturated by repeated passages through a fire-polished constricted Pasteur pipette. Dissociated tissues were allowed to settle for 3 min. Supernatants were transferred to a fresh tube, and were centrifuged at 1200 g for 3 min. Pellets were resuspended in NSC basal media with a proliferation supplement (Stem Cell Technologies, Vancouver, Canada), and 20 ng/mL epidermal growth factor (EGF, Invitrogen, Carlsbad, CA, USA). NSCs were plated on poly-D-ornithine (Sigma-Aldrich, St. Louis, MO, USA) treated dishes at a density of 2.5×10^4 cells/mL. Cultures were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Culture medium was replaced every 3 days. NSCs were used for experiments after 2–3 passages.

let-7a and siRNA TLX experiment

let-7a mimics, inhibitors, and siTLX were purchased from Ambion (Austin, TX, USA); they were mmu-*let-7a*-5p (*let-7a* mimic) (cat. #4464066) and *let-7a* inhibitor (cat. #4464066; assay ID MH10050). For RNA duplex transfection, 20 nM solutions in Opti-MEM (Sigma, St. Louis, MO, USA) were combined with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 15 min, the mixtures were added to the cells incubated for 72 h.

Measurement of neurosphere size

Images of neurosphere cultures were taken using an inverted microscope (Olympus microscopy BH3, Tokyo, Japan). The magnification (10×) ensured coverage of a significant area of each well of the 24-well plates. Image analysis software (Image J) was used to evaluate the size of neurospheres.

Reverse-transcription PCR

To examine the expression of *TLX*, *DCX*, and *c-Myc* in NSCs, RT-PCR was performed using specific primers. Briefly, the cell pellets were lysed with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). PCR was conducted by the following manual's conditions, and the following primers (5' to 3'): *TLX*, forward (F): GGC TCT CTA CTT CCG TGG ACA, reverse (R): GTC AGT ATT CAT GCC AGA TAC AGC CAG TG; *DCX*, (F): AAT CCC AAC TGG TCT GTC AAC, (R): GTT TCC CTT CAT GAC TCG GCA; *c-Myc*, (F): TCA AGA GGC GAA CAC ACA AC, (R): GGC CTT TTC ATT GTT TTC CA, and *GAPDH*, (F): ACA GTC CAT GCC ATC ACT GCC, (R): GCC TGC TTC ACC ACC TTC TTG. GAPDH was used as an internal control.

Real-time quantitative-PCR (SYBR and TaqMan assays)

For quantitative analysis of *let-7a*, reverse transcription was first performed using the TaqMan Micro RNA Reverse Transcription Kit (Takara, Otsu, Shiga, Japan) with total RNA samples of 10 ng. PCR was then performed according to the manufacturer's instructions using the TaqMan Universal PCR Master Mix, No Amp Erase UNG (Applied Biosystems, Foster City, CA, USA); PCR amplification was carried out in an ABI 7500 Real-Time PCR cycler (Bio-Rad, Philadelphia, PA, USA). We used the following primers (5' to 3'): *let-7a*, (F): GCG CCT GAG GTA GTA GGT TG, (R): CAG TGC AGG GTC CGA GGT; and *U6*, (F): CTC GCT TCG GCA GCA CAT ATA CT, (R): ACG CTT CAC GAA TTT GCG TGT C. The *let-7a* levels were normalized to the internal control *U6*.

Western blotting

The NSCs were homogenized in lysis buffer and centrifuged (12000 g at 4°C) for 15 min. Equal amounts of protein (30 μg) from the supernatants were separated on a 10% acrylamide gel, and the proteins were electrotransferred onto nitrocellulose membranes. After blocking, the membranes were incubated with primary antibody at 4°C. The primary antibodies included anti-DCX (1:2000, Millipore, Billerica, MA, USA), anti-NeuN (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and anti-β-actin (1:2000, Santa Cruz Biotechnology) antibodies. The membranes were then incubated with secondary antibodies. The blots were rinsed, and protein bands were visualized using an enhanced chemiluminescence detection system (Amersham, Pittsburgh, PA, USA).

Immunocytochemical analysis

The NSCs were incubated with primary antibody overnight at 4°C (goat anti-DCX; 1:200; Millipore). Cell samples were then incubated with a rhodamine-conjugated donkey anti-goat antibody (1:200, Jackson ImmunoResearch, West Grove, PA, USA) for 2 h at room temperature (RT). The NSCs were washed 3 times again, for 3 min each with PBS. The cells were then counterstained with 1 μg/mL 4', 6-diamidino-2-phenylindole (DAPI; 1:100; Invitrogen, Carlsbad, CA, USA) for 10 min at RT and were photographed using a confocal microscope (Carl Zeiss, Thornwood, NY, USA).

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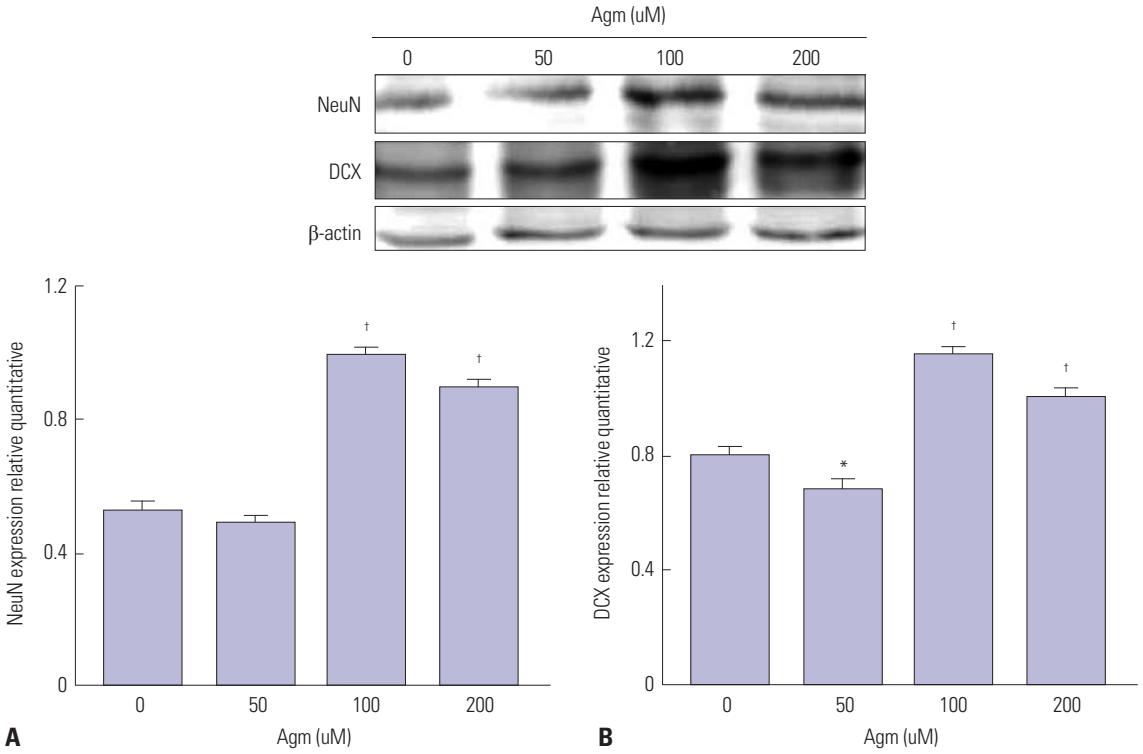

Fig. 1. Alterations in NeuN and DCX protein levels in NSCs by agmatine treatment. (A) The protein levels of NeuN increased in the agmatine treatment group. In particular, the 100 μM agmatine treatment significantly increased NeuN protein levels in NSCs. (B) DCX protein levels increased in the agmatine treatment group. Similar to NeuN, 100 μM agmatine significantly increased DCX protein levels in NSCs. β-actin was used as an internal control. Data are expressed as mean±SEM. *p<0.05, †p<0.001 compared to agmatine 0 uM group. Agm, agmatine treatment; NSCs, neural stem cells.

Statistical analysis

All calculations were conducted using SPSS 18.0 software (IBM Corp., Armonk, NY, USA). Data are expressed as mean±SEM. Significance of intergroup differences was determined by one-way analysis of variance followed by Bonferroni post hoc multiple-comparison tests. Each experiment included at least three repeats per condition. Differences with a p value less than 0.05 were considered statistically significant.

RESULTS

Alterations of NeuN and DCX expression in NSCs after agmatine treatment

We conducted western blot analyses of the expression of neuronal markers (NeuN[28] and DCX[29]) in NSCs after agmatine treatment (Fig. 1). Treatment with agmatine (100, 200 μM) increased expression of NeuN (Fig. 1A) and DCX (Fig. 1B), while 50 μM agmatine treatment showed a small decrease of DCX protein level (Fig. 1B). Specifically, 100 μM agmatine treatment visibly increased the protein levels of NeuN (Fig. 1A) and DCX (Fig. 1B). On the basis of these findings, we chose 100 μM agmatine for the following experiments with an assumption that 100 μM agmatine would promote neuronal differentiation in NSCs.

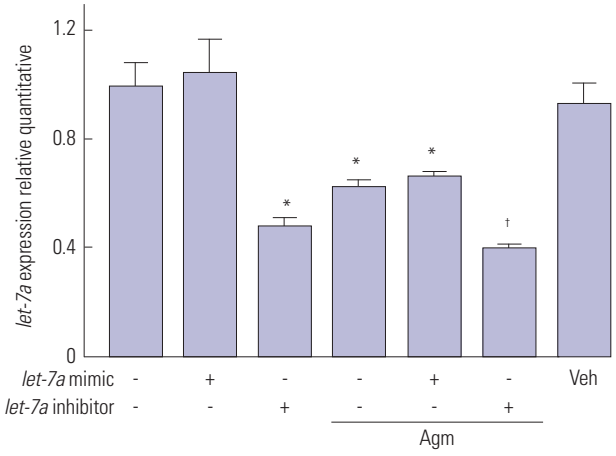

Fig. 2. Effects of alterations of *let-7a* levels in neural stem cells. A TaqMan assay was performed to check *let-7a* level. The level of *let-7a* decreased in the agmatine group compared with the *let-7a* mimic group. U6 was used as an internal control. Data are expressed as mean±SEM. *p<0.05, †p<0.001 compared to normal control group. *let-7a* mimic, *let-7a* mimic treatment/*let-7a* overexpression group; *let-7a* inhibitor, *let-7a* inhibitor treatment/*let-7a* suppression group; Agm, 100 μM agmatine treatment group; Veh, Lipofectamine treatment group.

let-7a levels in NSCs after agmatine treatment

In NSCs, we confirmed *let-7a* expression by a TaqMan assay (Fig. 2). Agmatine treatment decreased *let-7a* expression in NSCs compared with the normal control group. Although a few differences were observed between agmatine treatment and

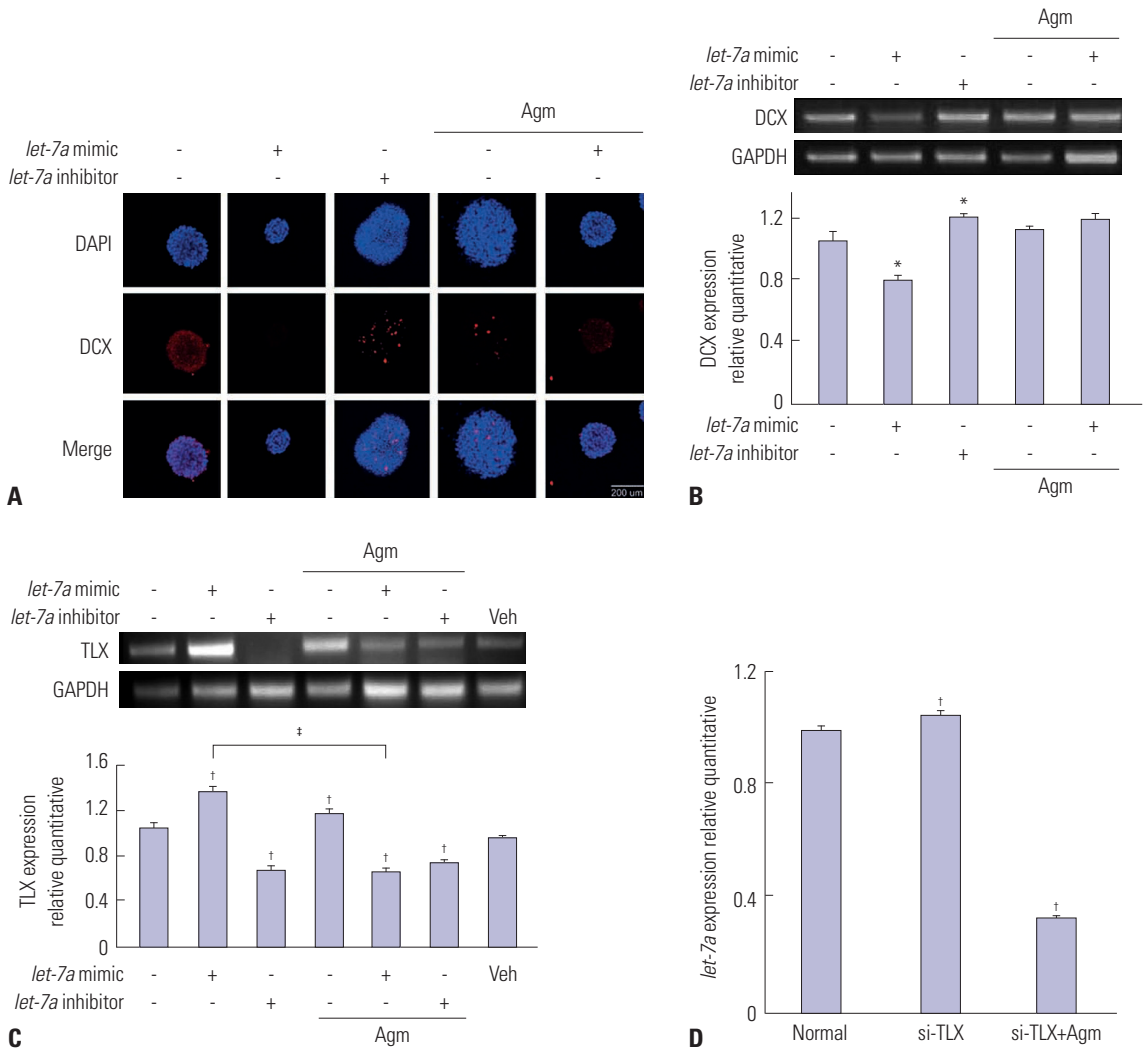

Fig. 3. The expression of DCX and TLX mRNA levels in neural stem cells. (A) Immunochemical images indicated that DCX-positive cells decreased in the *let-7a* mimic group. Scale bar: 200 μm. NC: the normal control group, 4', 6-diamidino-2-phenylindole (DAPI): blue, DCX: red. (B) Under agmatine treatment, the *let-7a* suppression group (*let-7a* inhibitor group) expressed lower DCX mRNA levels. GAPDH was used as an internal control. (C) The *let-7a* mimic group with agmatine had decreased TLX mRNA expression compared to the *let-7a* mimic group. GAPDH was used as an internal control. (D) Inhibition of TLX expression by agmatine decreased *let-7a* levels. U6 was used as an internal control. Data are expressed as mean±SEM. *$p<0.05$, †$p<0.001$ compared to normal control group, ‡$p<0.001$ compared to *let-7a* mimic group. *let-7a* mimic, *let-7a* mimic treatment/*let-7a* overexpression group; *let-7a* inhibitor, *let-7a* inhibitor treatment/*let-7a* suppression group; Normal, normal control group/no treatment; Agm, 100 μM agmatine treatment group; siTLX, TLX silencing group; siTLX+Agm, TLX silencing with agmatine treatment group.

agmatine treatment along with *let-7a* overexpression (the same with *let-7a* mimic treatment), we found that agmatine dramatically reduced *let-7a* levels in spite of *let-7a* mimic treatment (Fig. 2), indicating that agmatine negatively regulates *let-7a* expression in NSCs.

Changes in DCX expression in NSCs after agmatine treatment during *let-7a* overexpression

Immunochemical images of DCX indicated that *let-7a* overexpression inhibited the expression of DCX (Fig. 3A). Agmatine treatment increased the number of DCX-positive cells in both the *let-7a* overexpression state (*let-7a* mimic treatment) and the normal state (Fig. 3A). Fig. 3B demonstrates that *let-7a* overexpression (*let-7a* mimic treatment) in NSCs decreased DCX mRNA levels compared with the normal control group (Fig. 3B). Agmatine treatment group shows DCX mRNA induction that occurred with *let-7a* overexpression (Fig. 3B). Our results suggest that DCX, a marker for immature neurons, was downregulated under *let-7a* overexpression, whereas DCX expression was increased under *let-7a* overexpression in agmatine treatment group (Fig. 3).

Relationship between TLX expression and *let-7a* levels in NSCs

TLX mRNA levels are associated with self-renewal and proliferation[30] of NSCs. We found that *let-7a* overexpression (*let-7a* mimic treatment) increased TLX expression (Fig. 3C). Although the expression of TLX was increased by agmatine in NSCs, it

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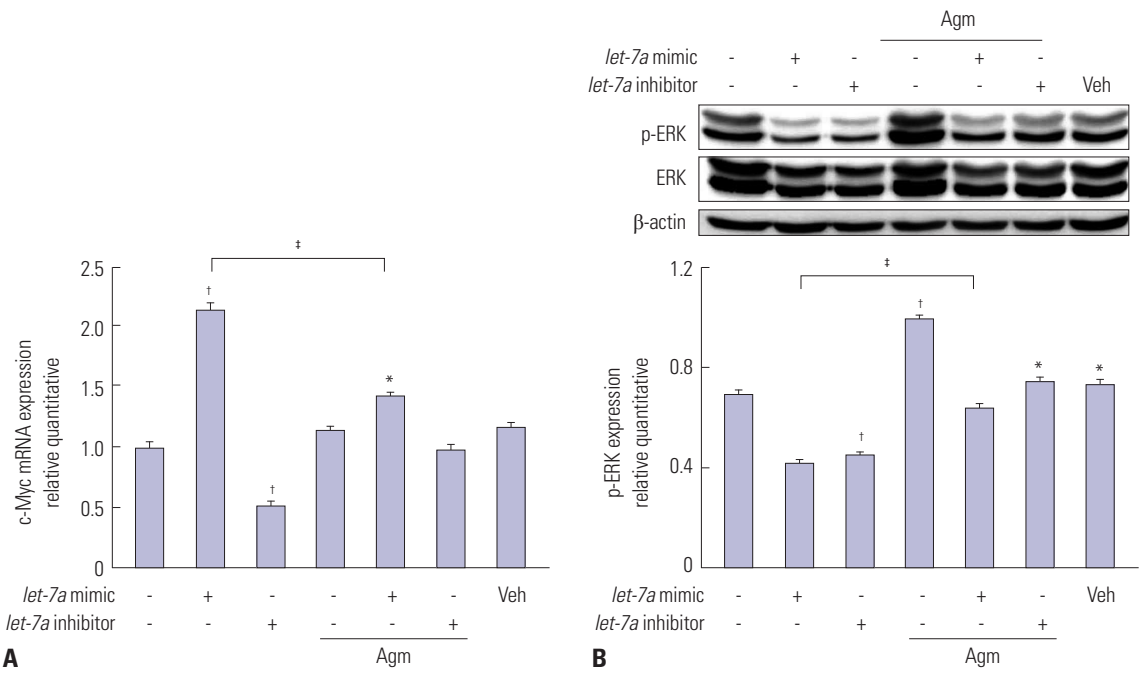

Fig. 4. Assessment of c-Myc mRNA and ERK phosphorylation in neural stem cells. (A) c-Myc mRNA expression was reduced in the *let-7a* mimic treatment with agmatine group in comparison with *let-7a* mimic group. β-actin was used as an internal control. (B) ERK phosphorylation significantly increased in the *let-7a* mimic treatment with agmatine group compared to *let-7a* mimic group. GAPDH was used as an internal control. Data are expressed as mean±SEM. *p<0.05, †p<0.001 compared to normal control group, ‡p<0.05 compared to *let-7a* mimic group. *let-7a* mimic, *let-7a* mimic treatment/*let-7a* overexpression group; *let-7a* inhibitor, *let-7a* inhibitor treatment/*let-7a* suppression group; Agm, 100 μM agmatine treatment group; Veh, Lipofectamine treatment group; p-ERK, ERK phosphorylation.

was noticeably reduced in agmatine treatment group under *let-7a* mimic treatment in comparison with the *let-7a* overexpression group (Fig. 3C). In addition, we observed a slight increase in TLX mRNA expression after the *let-7a* inhibitor treatment, in comparison with the *let-7a* overexpression group under agmatine treatment conditions (Fig. 3C). In the TLX knockdown group, *let-7a* expression increased slightly, whereas the agmatine co-treatment group showed a marked decrease in *let-7a* expression (Fig. 3D).

Change of c-Myc expression in NSCs

Expression of c-Myc mRNA in NSCs was measured using real-time quantitative RT-PCR (Fig. 4A), since c-Myc plays a crucial role in proliferation and self-renewal[31] of NSCs. We observed increased expression of c-Myc in the *let-7a* overexpression group (*let-7a* mimic treatment) (Fig. 4A). After agmatine treatment, c-Myc mRNA expression was slightly increased. However, the *let-7a* overexpression with agmatine treatment reduced the expression of c-Myc (Fig. 4A).

Change of the ERK pathway of NSCs

In order to determine signaling pathway affected by agmatine and *let-7a*, we measured ERK phosphorylation which is related to NSC proliferation (Fig. 4B).[12] Activation of ERK, as indicated by the level of phosphorylated ERK protein, was reduced in NSCs after *let-7a* mimic treatment. Agmatine increased the activation of ERK in NSCs, whereas the expression of ERK was decreased by *let-7a* overexpression (Fig. 4B). Given the fact that ERK activation is important for proliferation and self-renewal of NSCs,[32] our results indicated that agmatine may reverse the decrease of ERK phosphorylation, induced by *let-7a*.

DISCUSSION

Understanding of NSC proliferation, self-renewal, and differentiation is necessary to enhance the therapeutic efficacy of NSCs.[33] Agmatine has multiple roles in several biological processes.[34,35] In the present study, we found several lines of evidence indicating that agmatine can enhance NSC differentiation into neurons by modulating expression of *let-7a*. 100 μM agmatine was found to promote differentiation by upregulation of NeuN[28] and DCX.[29] Since *let-7a* was reduced in NSCs when treated with agmatine, our results suggest that agmatine may negatively regulate *let-7a* in NSCs. DCX is regarded as a marker of neurogenesis,[36] the findings therefore suggest that agmatine promotes NSC differentiation into neurons: it is quite possible that agmatine may boost neuronal differentiation by inhibiting *let-7a* expression.

Previous studies have demonstrated that underexpression of *let-7a* attenuates the proliferation and self-renewal of NSCs.[18] TLX is necessary for maintaining the proliferative potential of NSCs and neurogenesis.[37] In the present study, we suggest that agmatine may enhance neurogenesis by negatively regu-

lating expression of *let-7a* and TLX, and it may inhibit NSC proliferation by attenuating the increased TLX level in *let-7a* overexpression conditions.[38]

c-Myc regulates the genes involved in self-renewal, and proliferation[39] of NSCs, and the *let-7* family is known to regulate cell proliferation by targeting c-Myc.[40] In our present study, overexpression of *let-7a* upregulated the expression of c-Myc, which is known to sustain the stemness of stem cells targeted by the *let-7* family.[41] These findings imply that agmatine may reduce c-Myc via controlling *let-7a*, thereby influencing self-renewal and proliferation of NSCs.

It has been demonstrated that agmatine regulates neuroprotection and increases neuronal differentiation in NSCs by activating ERK1/2.[12] In our present study, we demonstrated that *let-7a* overexpression in NSCs decreases ERK phosphorylation, thus indicating that agmatine promotes ERK activation by negatively regulating *let-7a*, which is involved in neuronal differentiation and cell survival of NSCs.

In conclusion, we found four main findings in the present study. 1) Agmatine increased the expression of DCX and NeuN in NSCs. 2) Agmatine decreased the expression of *let-7a* in NSCs. 3) Agmatine attenuated the *let-7a*-induced upregulation of TLX and c-Myc. 4) Agmatine ameliorated the *let-7a*-induced ERK inactivation in NSCs. Therefore, we suggest that agmatine manipulates the mechanism linking *let-7a*, and induce neuronal differentiation for treatment of CNS disorders.

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